

Transmission of replication-defective Sindbis helper vectors encoding capsid and envelope proteins

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Abstract

A green fluorescent protein (gfp)-encoding derivative of the replication-defective Sindbis helper vector DHBB was constructed in order to study the rate of packaging of the helper vector. The results show that despite lacking a ‘packaging sequence’, this vector is co-packaged about 1/300th the rate of packaging of single RNAs containing a packaging sequence. This helps explain the frequent observation of recombinant, replication-competent viruses when using first generation Sindbis packaging systems. Because of their sensitivity, GFP-encoding reporters like the one described here will be useful for measuring transfer of helper RNAs in improved alpha virus packaging systems. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Green fluorescent protein; Sindbis helper vector; Packaging

Vectors derived from alphavirus replicons provide a convenient method for amplifying vector RNA in eukaryotic cells and obtaining high levels of expression of inserted genes (Xiong et al., 1989; Liljestrom and Garoff, 1991; Frolov et al., 1996; Garoff and Li, 1998). The addition of ‘helper’ vectors encoding alphavirus capsid and envelope proteins allows packaging of replicase-encoding vector RNA in alphavirus particles that can infect a variety of cell types. In order to avoid packaging of the helper vector, which leads to increased risk of recombination leading to replica-

tion-competent virus, defective helper vectors were constructed that lack ‘packaging sequences’ (Bredenbeek et al., 1993). Although initial reports indicated that optimized defective helper vectors were not detectably transmitted, the frequent observation of replication-competent recombinant viruses suggested that transmission was occurring below the level of detection.

To facilitate more sensitive detection of rate of transfer of helper vectors, a GFP reporter was added to the DHBB defective helper vector (Bredenbeek et al., 1993) downstream of a duplicated subgenomic promoter (Fig. 1). The DHBB vector was obtained from Invitrogen Corp. (Carlsbad, CA), and was confirmed by sequencing to carry the 5′ sequence of Sindbis virus, rather than the

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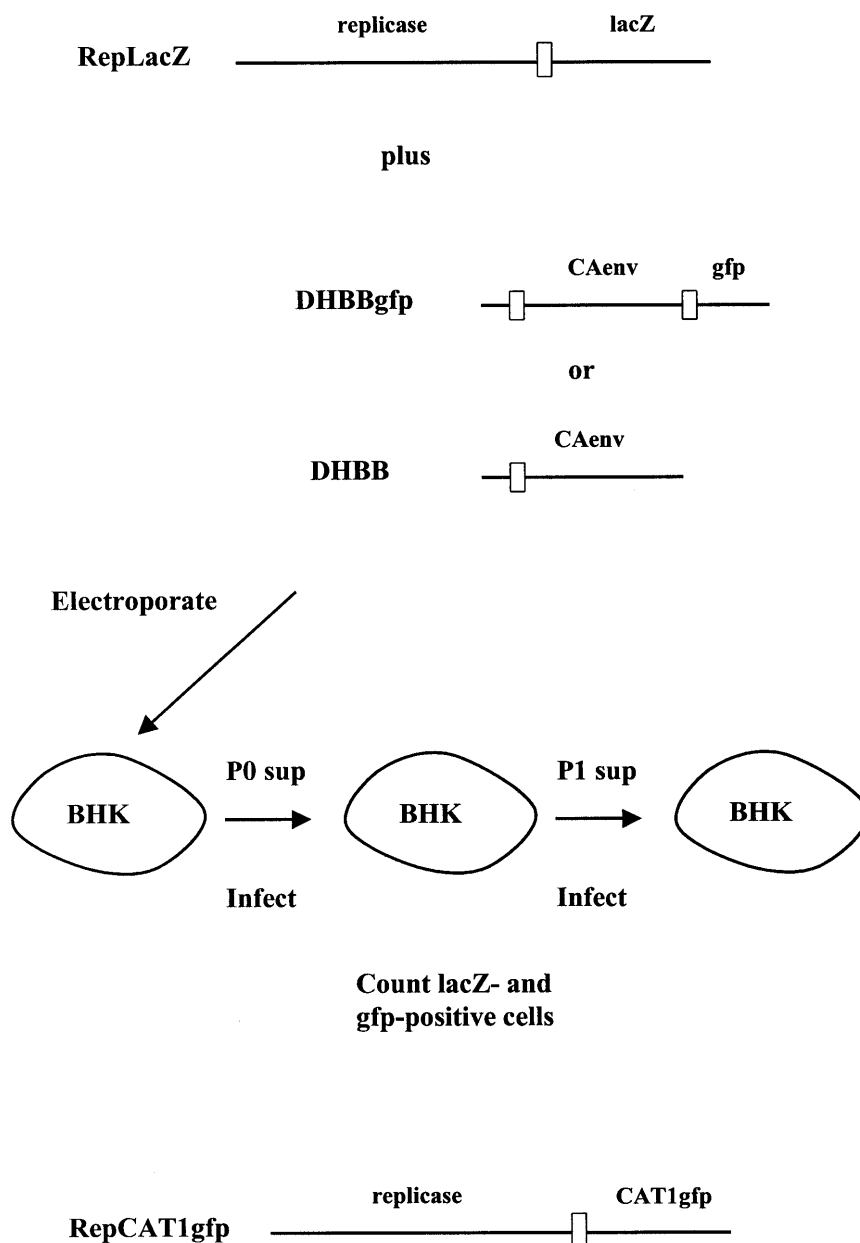


Fig. 1. Schematic diagram of vectors and protocol for electroporation and infection of BHK cells. RepLacZ denotes RNA transcribed in vitro from plasmid pSinRep5LacZ purchased from Invitrogen (Carlsbad, CA). DHBB-gfp denotes RNA transcribed from a derivative of pDHBB containing, after the stop codon of Sindbis envelope, a duplicated subgenomic promoter (positions 7498–7613 from pSinRep5) fused to the enhanced green fluorescent protein gene from Clontech (Palo Alto, CA). Electroporation was done as described (Kazachkov et al., 2000). P0 sup, passage 0 supernatant. P1 sup, passage 1 supernatant. CA, capsid. env, envelope. □, subgenomic promoter. RepCAT1gfp denotes RNA from pSinRep5CAT1gfp vector.

tRNA sequence in helper vectors derived from defective interfering particles that are known to package more efficiently (Bredenbeek et al., 1993). BHK cells were co-electroporated with RNA from the original or gfp-containing DHBB vector plus RNA from a vector encoding Sindbis replicase and beta-galactosidase (pSinRep5LacZ, Invitrogen, Carlsbad, CA). As expected, the electroporated cells expressed both beta-galactosidase and gfp and produced infectious particles containing the replicase-lacZ RNA. Filtered (0.45 μ m) supernatants from cells receiving the modified helper vector had a titer on BHK cells of about 10^5 lacZ-inducing units/ml, approximately 3-fold lower than obtained with the unmodified DHBB helper (Table 1).

Although filtered supernatants from electroporated cells might not be expected to contain helper vector RNA since this vector lacks a packaging sequence (Weiss et al., 1989; Frolova et al., 1997), nevertheless they transferred gfp expression to fresh BHK cells with a titer of 320 gfp-infectious units/ml (Table 1). Cells expressing gfp must have received replicase RNA as well as gfp RNA since small numbers of DHBB-gfp vector RNA molecules do

not produce detectable amounts of gfp, and the DHBB vector replicates only in the presence of alphavirus replicase. It is likely that gfp-expressing cells were infected with particles that contained co-packaged replicase plus helper RNA, as co-packaging is known to occur with Sindbis vectors (Geigenmuller-Gnirke et al., 1991). If gfp-expression resulted from co-packaging, the ratio of gfp to lacZ infectious units implies that the helper vector was co-packaged at a rate about 1/300th the rate at which replicase vector was packaged alone. Alternatively, gfp expression could have resulted from infection with separate particles encoding replicase and gfp. If this were the case, the data would imply that the helper vector was packaged at nearly 1/30th the rate of replicase vector, taking into account the multiplicity of infection of the replicase vector. However, this seems less likely since the number of gfp-positive cells declined linearly with dilution (Table 1), whereas it would be expected to decline with the square of dilution if infection with two particles were required. A third possibility is that gfp-expressing cells were infected with recombinant vector encoding replicase plus gfp.

A repeat experiment electroporating BHK cells with the DHBB-gfp vector plus pSinLacZ RNA gave very similar results: a lacZ titer of 3.6×10^4 /ml and a gfp titer of 120/ml.

If DHBB-gfp RNA was co-packaged with replicon RNA, then lacZ infectious particles should be produced in subsequent rounds of infection. To see if this was the case, BHK cells were infected with 2000 lacZ infectious units from the supernatant of electroporated cells (passage 0 supernatant). Passage 1 supernatants were collected 36 h later, filtered, and assayed on fresh BHK cells. The passage 1 supernatant derived from the DHBB-gfp vector contained 240 lacZ infectious units/ml and 40 gfp infectious units/ml, while the passage 1 supernatant derived from the original DHBB vector contained 80 lacZ infectious units/ml (Table 1). This shows that RNA encoding gfp and alphavirus structural genes was packaged after the first round of infection and suggests that co-packaging/recombination may be slightly more frequent with the modified DHBB-gfp helper than with the original DHBB vector.

The assay for lacZ and gfp positive cells showed

Table 1

LacZ and gfp infectious units in serial dilutions of supernatant of BHK cells electroporated with SinRepLacZ plus helper RNA (passage 0 sup), or supernatant of BHK cells infected with passage 0 supernatant containing 2000 lacZ infectious units (passage 1 sup)^a

LacZ and gfp infectious units		
Helper vector	Passage 0 sup	Passage 1 sup
DHBBgfp	lacZ/gfp	lacZ/gfp
100 μ l	TMTC/32	24*/4*
10 μ l	TMTC/2	2*/0
1 μ l	91/0	0/0
0.1 μ l	11/0	nd
Titer	9.1×10^4 /320	240/40
DHBB		
100 μ l	TMTC	8
10 μ l	TMTC	0
1 μ l	TMTC	0
0.1 μ l	31	nd
Titer (per ml)	3.1×10^5	80

^a *Includes clusters of lacZ and gfp positive cells (see Fig. 2), each cluster being counted as a single infectious unit. TMTC, too many to count. nd, not done.

single lacZ-positive cells as well as clusters of cells co-expressing lacZ and gfp after infection with passage 1 supernatants (Fig. 2, panels A–C). Co-expression of lacZ and gfp was detected by first recording gfp fluorescence and then staining for lacZ, since the latter procedure involves fixation that inhibits gfp fluorescence. Clusters of cells expressing lacZ (or gfp) were not seen in passage 1 supernatants derived from the DHBB helper or in assays of passage 0 supernatants. The new appearance of clusters of gfp-positive cells suggests a change in the gfp-encoding vector causing it to

be transferred with high efficiency to neighboring cells. For example, the helper vector could have picked up a packaging sequence or the gfp gene could have been transferred to the replicase vector. Rapid spread of a recombinant gfp-encoding vector would also explain why the ratio of gfp to lacZ infectious units was nearly 50-fold higher in the passage 1 supernatant than in the passage 0 supernatant ($40/240 = 1/6$ versus $320/91000 = 1/284$).

We attempted to confirm that helper vector RNA was packaged by analysis of radio-labeled RNA from electroporated cells. However, as re-

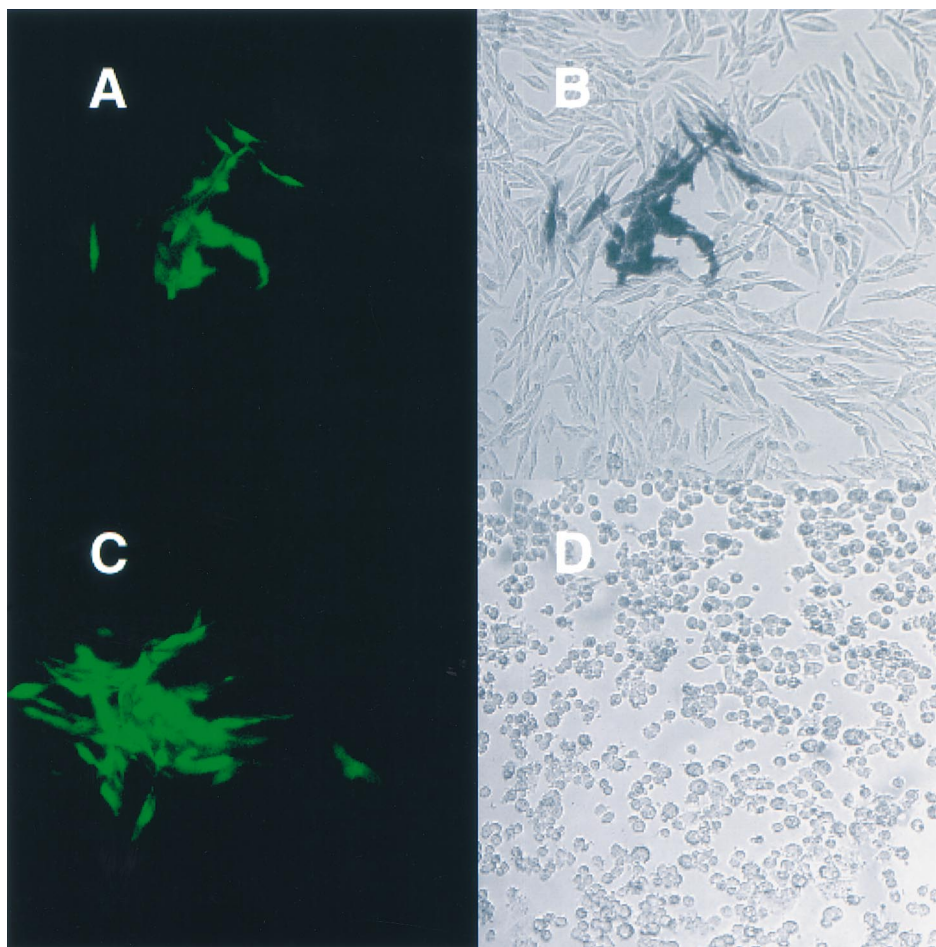


Fig. 2. Photomicrographs of BHK cells infected with P1 supernatants derived from the PO supernatants from cells electroporated with DHBB-gfp and SinRepLacZ RNA. Panels A and C show clusters of cells exhibiting green fluorescence detected with a Zeiss 410 laser scanning microscope using 488 nm excitation and 515–565 nm emission filters. Panel B shows the same field as panel A following staining for lacZ as recommended in the pSinLacZ manual from Invitrogen. Panel D shows cells exhibiting cytopathic effect of recombinant, replication competent virus (see text).

ported previously (Bredenbeek et al., 1993), the amount of DHBB RNA in electroporated cells was much lower than the amount of replicase RNA, and no DHBB RNA was detected in electroporation supernatants (data not shown).

Transfer of DHBB RNA was much more pronounced when a replicase vector was used encoding the ecotropic murine leukemia virus (MLV) receptor, mCAT1, fused to gfp, pSinRep5CAT1gfp (Kazachkov et al., 2000) (lower part of Fig. 1). BHK cells were co-electroporated with pSinRep5CAT1gfp RNA plus DHBB RNA and 2000 CATgfp-infectious units from the (passage 0) filtered supernatant were used to infect fresh BHK cells. The filtered supernatant of the infected BHK cells (passage 1 supernatant) should not be infectious in BHK cells unless DHBB RNA was carried over from passage 0 and produced Sindbis virions. However, the passage 1 supernatant had a titer of 1.4×10^4 gfp-inducing units/ml on BHK cells (Table 2). This is 60–175-fold greater than the titer of the lacZ vector in passage 1 supernatants (Table 1), which suggests that the pSinCAT1gfp vector induces co-packaging or recombination with DHBB RNA more often than the pSinLacZ vector. The reason for this is not understood; it is not a length effect since the CAT1gfp insert is shorter than lacZ (~ 2500 versus 3000 base pairs); nor was any sequence homology noted between the CAT1gfp insert and the DHBB vector.

Spread of the CAT1gfp vector could be enhanced by taking advantage of the fact that cells expressing the CAT1 receptor formed syncytia with cells expressing a fusogenic form of the MLV envelop on their surface (Kazachkov et al., 2000). When 2000 CAT1gfp-infectious units from the passage 0 electroporation supernatant were used to infect BHK cells expressing MLV envelope, large syncytia expressing gfp were detected. Supernatant of these cells (passage 1 supernatant) had a titer of 1.6×10^6 gfp-inducing units on plain BHK cells. Infection of plain BHK cells in this system is mediated by Sindbis virions since supernatants of BHK cells electroporated with the CAT1gfp vector alone did not transfer gfp to BHK cells. The nearly 100-fold higher titer of Sindbis virions containing CAT1gfp vector RNA in this experiment, compared to passage 1 supernatants from plain BHK cells, is likely due to spread of replicon RNA via CAT1-MLV envelope-mediated fusion to cells that were infected with DHBB helper RNA alone.

In rare experiments, cytopathic effect (CPE) characteristic of replication competent Sindbis virus was observed in passage 1 or higher level passages (Fig. 2, panel D). After further passage, the titers of such supernatants, measured by their ability to induce characteristic CPE on BHK cells, reached or exceeded 10^7 /ml, strongly suggesting that they contained replication competent viruses due to recombination. Recombination between

Table 2

Gfp infectious units in serial dilutions of passage 0 supernatant of BHK cells electroporated with SinRepCAT1gfp plus DHBB helper RNA, or passage 1 supernatants from BHK cells (or BHK-MLVenv cells) infected with passage 0 supernatant containing 2000 gfp infectious units^a

Gfp infectious units			
	Passage 0 sup of BHK cells	Passage 1 sup of BHK cells	Passage 1 sup of BHK-MLVenv cells
100 μ l	nd	TMTC	nd
10 μ l	nd	TMTC	nd
1 μ l	TMTC	14	TMTC
0.1 μ l	TMTC	nd	TMTC
0.01 μ l	12	nd	16
0.001 μ l	2	nd	nd
Titer (per ml)	1.2×10^6	1.4×10^4	1.6×10^6

^a BHK-MLVenv cells express MLV envelope and fuse with cells expressing CAT1 (Kazachkov et al., 2000). Titrations for gfp infectious units were done on BHK cells; nearly identical results were obtained when titrations were done on BHK-MLVenv cells.

alphaviruses, including recombination leading to replication competent virus, is well documented (Raju et al., 1995; Strauss and Strauss, 1997).

The experiments demonstrate that RNA from a DHBB vector with a duplicated subgenomic promoter plus gfp is co-packaged in Sindbis virion particles at a rate $\sim 1/300$ th that of pSinRepLacZ RNA. The rate of packaging of single DHBB RNA molecules may have been higher since only cells containing both helper and replicon RNAs were detected. Furthermore, this estimate does not take into account the fact that the amount of DHBB RNA in cells is much less than that of replicase-encoding RNA (Bredenbeek et al., 1993); therefore, on a per molecule basis the rate of packaging of helper RNA should be higher still. The titer of replicase vector in passage 1 supernatants was 60–175-fold higher when DHBB was used in conjunction with the pSinCAT1gfp vector rather than with the pSinLacZ vector, indicating that the replicase vector contributes in unexpected ways to the rate of co-packaging or recombination.

Previous results failed to detect packaging of DHBB RNA using a plaque assay as well as biochemical assays for transmitted helper RNA or protein (Bredenbeek et al., 1993). The plaque assay could have failed to detect packaged DHBB RNA because plaques require multiple rounds of infection which would be inhibited by high titers of packaged replicon RNA as a result of superinfection resistance. The plaque assay also relies on cytopathic effect (CPE) which is largely (though not entirely) a consequence of expression of Sindbis structural proteins (Frolov and Schlesinger, 1994). Since the DHBB vector does not replicate as well as replicase-containing vectors or other defective helpers (Bredenbeek et al., 1993), its RNA would be more difficult to detect and it would be expected to produce less structural protein, leading to less CPE. It is noteworthy that a different helper vector, DH(26S)5'SIN, whose RNA was packaged at about 1/10th the rate of replicase RNA, also did not produce detectable plaques (Bredenbeek et al., 1993), further indicating that the plaque assay could have missed transmission of DHBB. In contrast, our assay for transmission of helper RNA is extremely sensitive

and reliably detects transfer of replicase plus helper RNAs to single cells.

In order to reduce the chance of generation of replication-competent virus by recombination, second generation packaging systems have been developed in which capsid and envelope genes are produced from separate vectors and mutations have been introduced to block capsid protease function (Pushko et al., 1997; Polo et al., 1999; Smerdou and Liljestrom, 1999). While, in theory, these modifications should make generation of replication-competent virus orders of magnitude less likely, past experience with viral vector systems shows the importance of careful tests for intermediates in the process leading to replication-competence. Vectors analogous to the gfp reporters described here should be useful for sensitive detection of transfer of helper vectors in these packaging systems.

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